A single starfish Aurora kinase performs the combined functions of Aurora-A and Aurora-B in human cells

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Summary

Aurora, an essential mitotic kinase, is highly conserved during evolution. Most vertebrates have at least two Aurora kinases, Aurora-A and Aurora-B, which have distinct functions in the centrosome–spindle and inner centromere–midbody, respectively. However, some non-vertebrate deuterostomes have only a single Aurora. It remains to be verified whether the single Aurora performs the same functions as vertebrate Auroras A and B combined. We have isolated a cDNA of a single Aurora (ApAurora) from the echinoderm starfish, *Asterina pectinifera*, and show that ApAurora displays most features of both Aurora-A and Aurora-B in starfish oocytes and early embryos. Furthermore, ApAurora that is stably expressed in HeLa cells can substitute for both human Aurora-A and Aurora-B when either is reduced by RNAi. A single ApAurora thus has properties of both Aurora-A and Aurora-B in starfish eggs and HeLa cells. Together with phylogenetic analysis indicating that ApAurora forms a clade with all types of vertebrate Auroras and single Auroras of non-vertebrate deuterostomes, our observations support the idea that the single Aurora found in non-vertebrate deuterostomes represents the ancestor that gave rise to various types of vertebrate Auroras. This study thus provides functional evidence for phylogenetic considerations.

Key words: Aurora kinase, INCENP, Mitotic kinases, Molecular evolution, TPX2

Introduction

Accurate progression of mitosis (M phase) is ensured by mitotic kinases such as Cdc2, Plk1 and Aurora (Nigg, 2001; Taylor and Peters, 2008). Aurora is a family of serine/threonine kinases conserved from yeast to human. Whereas there is only a single type of Aurora in yeast (Ipl1 in budding yeast and Ark1 in fission yeast), some metazoans have at least two types of Aurora, Aurora-A and Aurora-B. Aurora-A and Aurora-B have highly similar sequences within the kinase domain, but show distinct subcellular localizations and functions (Carmena and Earnshaw, 2003; Giet et al., 2005; Keen and Taylor, 2004; Taylor and Peters, 2008). Aurora-A is localized on centrosome and spindle, and binds to and is activated by TPX2, a microtubule-binding protein that localizes Aurora-A to the spindle (Bayliss et al., 2003; Eyers et al., 2003; Kufer et al., 2002). Aurora-A regulates centrosome maturation, spindle assembly (Barr and Gergely, 2007; Marumoto et al., 2005) and probably mitotic entry through activation of Plk1 (Macurek et al., 2008; Seki et al., 2008). By contrast, Aurora-B is localized on the inner centromere from prometaphase to metaphase, and relocates into the midzone at anaphase onset, and finally into the midbody at cytokinesis (Ruchaud et al., 2007; Vader et al., 2006). Aurora-B localization depends on the formation of a chromosomal passenger protein complex containing inner centromere protein (INCENP), Survivin and Borealin, and the activation of Aurora-B depends on binding to INCENP (Jeyaprakash et al., 2007; Kelly et al., 2007; Rosasco-Nitcher et al., 2008; Ruchaud et al., 2007; Sessa et al., 2005; Vader et al., 2006). Aurora-B regulates kinetochore–microtubule attachment, the spindle assembly checkpoint, and cytokinesis (Jeyaprakash et al., 2007; Kelly et al., 2007; Rosasco-Nitcher et al., 2008; Ruchaud et al., 2007; Sessa et al., 2005). There are various substrates of Aurora-B, but phosphorylation of histone H3 on Ser10 is taken as an indicator of Aurora-B activity (Ruchaud et al., 2007).

Although properties of Aurora-A and Aurora-B are quite different, the social amoeba *Dictyostelium discoideum* has a single Aurora that displays the properties of both Aurora-A and Aurora-B. Hence, the *Dictyostelium* Aurora has been proposed to represent the ancestral kinase that gave rise to the different Aurora kinases in metazoans (Li et al., 2008). There are two Auroras in the protostomes *Drosophila melanogaster* and *Caenorhabditis elegans*. The fruit fly Aurora (DmAurora) and the nematode AIR-1 are classified as Aurora-A, whereas the fruit fly IAL and the nematode AIR-2 are classified as Aurora-B (Keen and Taylor, 2004; Nigg, 2001). However, sequence analyses show that DmAurora and IAL, as well as AIR-1 and AIR-2, are paralogous, and also that vertebrate Auroras are paralogous, rather than orthologous, to their counterparts in fruit fly and nematode (Brown et al., 2004). This implies that the duplication of the Aurora gene has occurred independently in vertebrates and protostomes. Consistently, in the phylogenetic tree, all vertebrate Auroras (types A, B and C) form a single clade that is distinct from those of the other organisms (Brown et al., 2004; Li et al., 2008). It is therefore plausible that a putative ancestral Aurora kinase for vertebrates could be found within deuterostomes.

Recent genome sequence analyses revealed that some non-vertebrate deuterostomes, including *Strongylocentrotus purpuratus* (sea urchin in echinoderms) (Sodergren et al., 2006),

Ciona intestinalis (ascidian in urochordates) (Dehal et al., 2002) and *Branchiostoma floridae* (amphioxus in cephalochordates) (Putnam et al., 2008), have only a single Aurora. The close evolutionary relationship of these organisms to vertebrates suggests that their single Aurora might represent the ancestral kinase of Aurora-A and Aurora-B in vertebrates. Indeed, on the basis of phylogenetic trees, it is proposed that all vertebrate Auroras evolved from a single urochordate ancestor, represented by the ascidian (Brown et al., 2004). However, these phylogenetic considerations seem difficult to reconcile with the very distinct subcellular localizations and functions of Aurora-A and Aurora-B. One possibility is that the single ancestral Aurora performs the same functions as Auroras A and B combined, and another possibility is that one or both of the vertebrate Auroras have gained fundamentally new and different functions from those of the ancestral Aurora.

Among cephalochordates, urochordates and echinoderms, the echinoderm starfish represents a model system in which molecular mechanisms of meiotic and mitotic cell cycle control have been extensively studied in oocytes and early embryos (Hara et al., 2009; Kishimoto, 2003; Nishiyama et al., 2010; Okano-Uchida et al., 2003; Okano-Uchida et al., 1998; Okumura et al., 1996; Ookata et al., 1992; Tachibana et al., 2008). Immature oocytes of the starfish are arrested at prophase of meiosis I, and reinitiation of meiosis is induced by the stimulus of the maturation-inducing hormone, 1-methyladenine (1-MeAde) (Kanatani et al., 1969). Once meiosis is reinitiated, meiosis I and II are completed in the absence of fertilization. Upon fertilization, eggs undergo early embryonic cycles. The genome has not yet been sequenced in any species of the starfish, but from many studies in the starfish *Asterina pectinifera* (renamed *Patiria pectinifera* in the 2007 NCBI Taxonomy Browser; http://www.ncbi.nlm.nih.gov/Taxonomy/), we have learned by experience that there is no subtype in many cell cycle regulators, including Cdc25 (Okumura et al., 1996), Wee1 (Okano-Uchida et al., 2003), cyclin A (Okano-Uchida et al., 1998) and cyclin B (Ookata et al., 1992). It can thus be anticipated that the starfish Aurora, if isolated, could demonstrate whether the single deuterostome Aurora can functionally play the dual roles of vertebrate Aurora-A and Aurora-B.

In the present study, we isolated one type of Aurora (ApAurora) from *A. pectinifera*. ApAurora displayed properties of both Aurora-A and Aurora-B in starfish oocytes and early embryos. In HeLa cells in which ApAurora was stably expressed, ApAurora was able to substitute for vertebrate Aurora-A and Aurora-B when either was knocked down by RNAi. In addition to these cell biological analyses, phylogenetic analysis indicated that ApAurora forms a higher order clade together with the single Auroras of sea urchin, amphioxus and ascidian, and a clade of vertebrate Auroras. It is thus probable that the single Aurora found in non-vertebrate deuterostomes represents the ancestral kinase that gave rise to vertebrate Aurora-A and Aurora-B.

Results

Identification of starfish Aurora

To investigate the functions of single Aurora, we first isolated an Aurora homolog from the starfish *A. pectinifera*. Only one type of

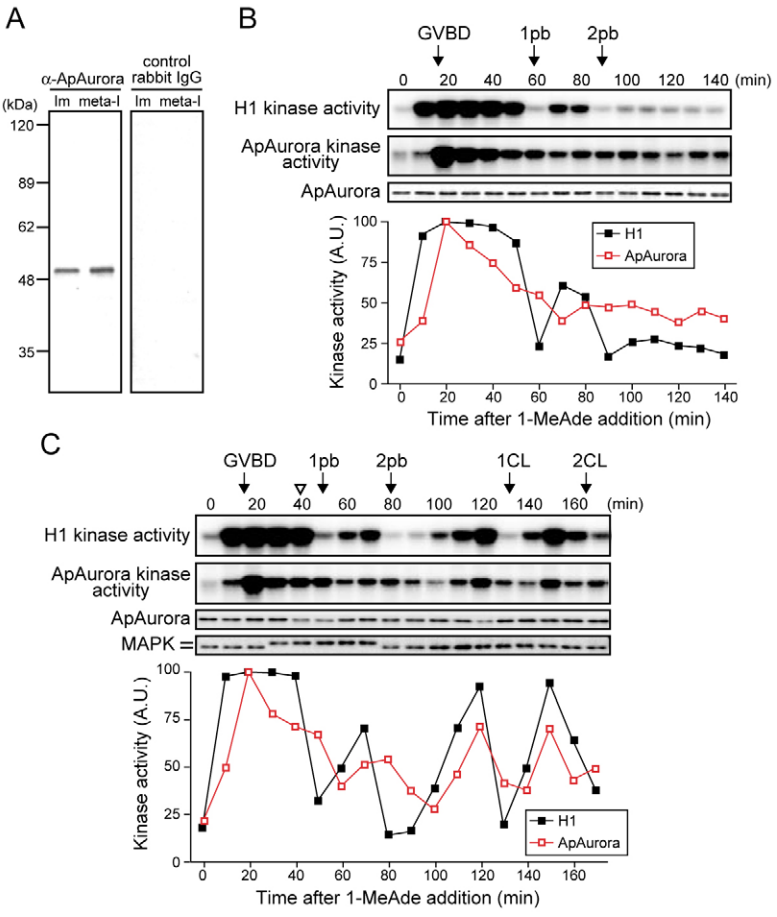

Fig. 1. Dynamics of changes in ApAurora protein levels and kinase activity in starfish meiotic and early embryonic cycles. (**A**) Specificity of the anti-ApAurora antibody against starfish Aurora. Extracts from immature oocytes (Im) or oocytes of metaphase of meiosis I (meta-I) were separated on a 9% SDS-PAGE gel and blots were probed with affinity-purified anti-ApAurora or control rabbit IgG, respectively. (**B**,**C**) Extracts were prepared from unfertilized (B) or fertilized (C) oocytes and eggs at 10 minutes intervals after 1-MeAde addition. Whole extracts and anti-ApAurora immunoprecipitates were assayed for H1 and ApAurora kinase activity, respectively. ApAurora and H1 kinase activities are quantified in the graphs beneath. ApAurora protein levels were examined by immunoblot with the anti-ApAurora antibodies. Transition from meiotic to embryonic cell cycle was monitored by immunoblot of MAPK (C). The active MAPK is essential for completion of meiotic cell cycle and its inactivation after meiosis II is necessary for initiation of embryonic cell cycle (see Hara et al., 2009). The upper and lower bands correspond to the active and inactive forms of MAPK, respectively. Arrows indicate the timing of GVBD, the first (1pb) and the second (2pb) polar body extrusion, and the first (1CL) and the second (2CL) cleavage. White arrowhead indicates the timing of insemination.

Aurora homolog was found in our screening of starfish cDNA libraries (see supplementary material Fig. S1), being consistent with our previous studies in which no subtypes were found in many cell cycle regulators (Kishimoto, 2003). Considering that in the genome of sea urchin, which is closely related to starfish in the phylum Echinodermata, only a single type of Aurora has been found and no subtypes in many cell cycle regulators (Fernandez-Guerra et al., 2006), we concluded that the starfish has only a single Aurora (named ApAurora).

ApAurora consists of 407 amino acids and has high similarity to human and *Xenopus* Aurora-A and Aurora-B in the kinase domain (see supplementary material Fig. S1). However, the N-terminal sequence is not quite similar to either Aurora-A or Aurora-B. It was thus difficult to determine which type of Aurora-A or Aurora-B is closer to ApAurora.

To examine protein dynamics of ApAurora in starfish meiotic and early embryonic cycles, we raised polyclonal antibodies against ApAurora (Fig. 1A). ApAurora protein levels remained constant throughout meiotic and early embryonic cycles (Fig. 1B,C). To examine the kinase activity of ApAurora, we performed an ApAurora kinase assay with anti-ApAurora immunoprecipitates using myelin basic protein as a substrate. ApAurora activity was very low in immature oocytes and abruptly increased at germinal vesicle breakdown (GVBD), with a slight delay after cyclin-B–Cdc2 activation. After GVBD, ApAurora activity decreased but was maintained at significantly elevated levels, and slightly fluctuated along with meiotic cycles (Fig. 1B). When maturing oocytes were inseminated at metaphase of meiosis I to proceed into embryonic cycles after meiosis II, ApAurora activity decreased soon after inactivation of MAPK and H1 kinase at completion of meiosis II and, thereafter, fluctuated almost in parallel with Cdc2 activity peaking at M phase (Fig. 1C). Thus, characteristics of ApAurora kinase activity displayed a hallmark feature of mitotic kinase.

ApAurora is implicated in entry into M phase, both as Aurora-A and Aurora-B

To examine the interdependency between ApAurora and mitotic kinases, we first confirmed that an Aurora-specific inhibitor, VX-680 (Harrington et al., 2004), suppressed the activity of ApAurora in vitro, but not of Cdc2 and Plk1 (see supplementary material Fig. S2A). To verify ApAurora inhibition by VX-680 in vivo, immature oocytes were preincubated with VX-680 and then treated with 1-MeAde (Fig. 2A). GVBD occurred almost normally with a slight delay of less than 2 minutes (data not shown) but, thereafter, ApAurora activity was undetectable at concentrations above 2 μM VX-680, indicating that VX-680 can inhibit ApAurora activity in vivo as well.

We then examined phosphorylation of histone H3 on Ser10 as a marker of Aurora-B activity in vivo (Ruchaud et al., 2007). Although the H3-Ser10 phosphorylation was observed in control meta-I oocytes, it was suppressed in the presence of VX-680 (Fig.

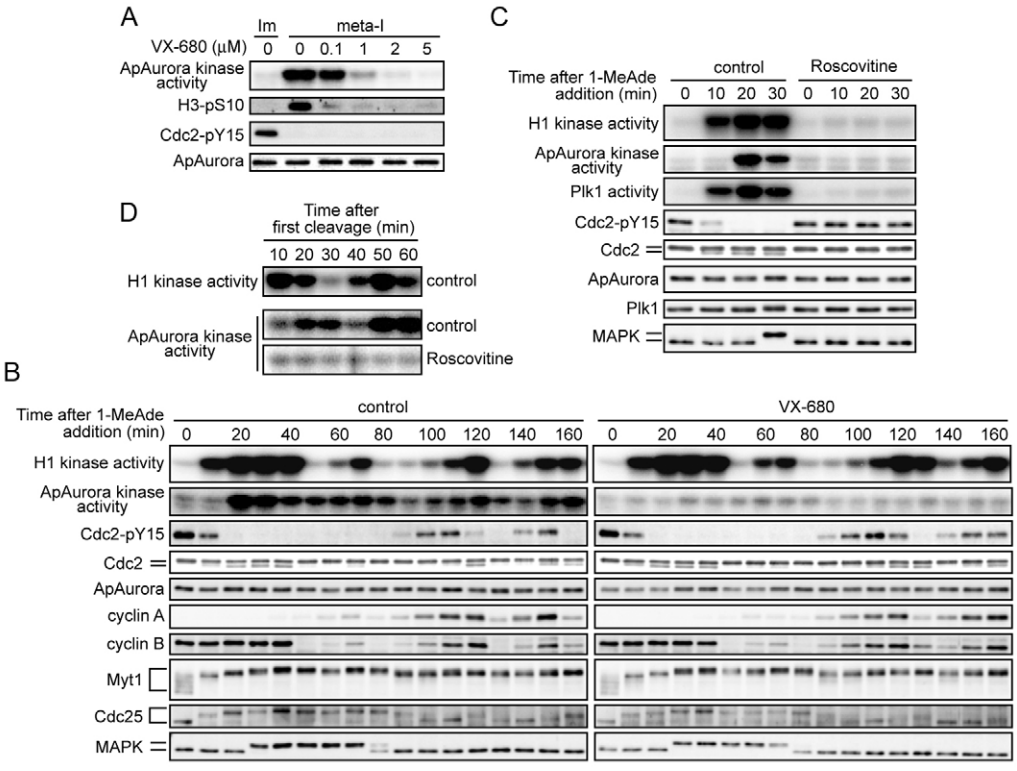

Fig. 2. ApAurora activity depends on Cdc2 activity, not vice versa, in starfish eggs. (**A**) ApAurora activity contributes to phosphorylation of histone H3 on Ser10. Immature oocytes were preincubated with VX-680 at indicated concentrations. Immature (Im) and metaphase of meiosis I (meta-I) oocytes were assayed. Dephosphorylation of Cdc2 on Tyr15 was used as a M-phase marker. (**B**) Cdc2 activity cycles in the absence of ApAurora activity throughout meiotic and early embryonic cycles. Immature oocytes were preincubated with DMSO (control) or 2 μM VX-680. Maturing oocytes were inseminated at meta-I. (**C**,**D**) ApAurora activation depends on Cdc2 activity. Immature oocytes were preincubated with DMSO (control) or 20 μM roscovitine (C). Maturing oocytes were inseminated at meta-I, followed by treatment with DMSO (control) or 20 μM roscovitine after the first cleavage (D). Cell cycle progression was monitored by immunoblots using anti-Cdc2-pY15, anti-Cdc2, anti-cyclin A, anti-cyclin B, anti-Myt1, anti-Cdc25, anti-Plk1 and anti-MAPK antibodies, and by Plk1 activity.

2A). This observation indicates that ApAurora plays the role of Aurora-B for phosphorylating histone H3-Ser10 in vivo.

As anticipated by the occurrence of GVBD, Cdc2 was normally activated after 1-MeAde addition in oocytes in which ApAurora activity was inhibited by VX-680 treatment (Fig. 2B). Thereafter, Cdc2 activity fluctuated through meiotic and early embryonic cycles in a similar manner as in control oocytes and eggs. Consistently, all assayed cell cycle regulators behaved similarly to those in controls (i.e. accumulation and disappearance of cyclin A and cyclin B proteins, activation and inactivation of MAPK, electrophoretic mobility shifts of Cdc25 and Myt1) (Fig. 2B). These observations indicated that ApAurora activity is not required for Cdc2 activation and inactivation throughout meiotic and early embryonic cycles. Conversely, when Cdc2 activity was inhibited by its specific inhibitor roscovitine (see supplementary material Fig. S2B), ApAurora was not activated at reinitiation of meiosis I nor at entry into early embryonic M phase (Fig. 2C,D). Thus, ApAurora activity is not required for Cdc2 activation, but Cdc2 activity is required for ApAurora activation.

Because Aurora-A is implicated in Plk1 activation in human cells (Macurek et al., 2008; Seki et al., 2008), we then examined their interdependency for activation both at reinitiation of meiosis I and entry into embryonic M phase. Plk1 kinase activity was measured in anti-Plk1 immunoprecipitates using α-casein as a substrate, as previously described (Okano-Uchida et al., 2003). When ApAurora activity was inhibited by VX-680 at meiotic reinitiation, activation of Plk1 occurred following that of Cdc2, although the level of Plk1 activity was significantly lower than that of the control (Fig. 3A). When eggs, after completion of meiosis II, were treated with VX-680 and then inseminated to proceed into embryonic cycles, Plk1 activation occurred following Cdc2 activation, but the level was significantly lower than that of the control (Fig. 3B). Moreover, in interphase, Plk1 activity was almost undetectable in VX-680-treated embryos. These observations indicate a partial, but not full, dependency of Plk1 activation on ApAurora activity.

By contrast, when Plk1 activity was prevented by injection of a neutralizing anti-Plk1 antibody (Okano-Uchida et al., 2003) or by treatment with BI 2536 (Steegmaier et al., 2007), a Plk1-specific inhibitor (see supplementary material Fig. S2C,D), at meiotic reinitiation, ApAurora was activated following Cdc2 activation although the level of ApAurora activity was higher than that of the control (Fig. 3C). When eggs, after completion of meiosis II, were treated with BI 2536 and then inseminated, ApAurora activation occurred normally, as in control eggs (Fig. 3B). These observations indicate that Plk1 activity is not required for ApAurora activation.

Taken together, the results indicate that ApAurora activation completely depends on activity of Cdc2, but not of Plk1, and conversely, that ApAurora activity is unnecessary for Cdc2 activation but partially necessary for Plk1 activation. Thus, ApAurora contributes as Aurora-A to Plk1 activation, and as Aurora-B to phosphorylation of histone H3 Ser10 in the starfish system.

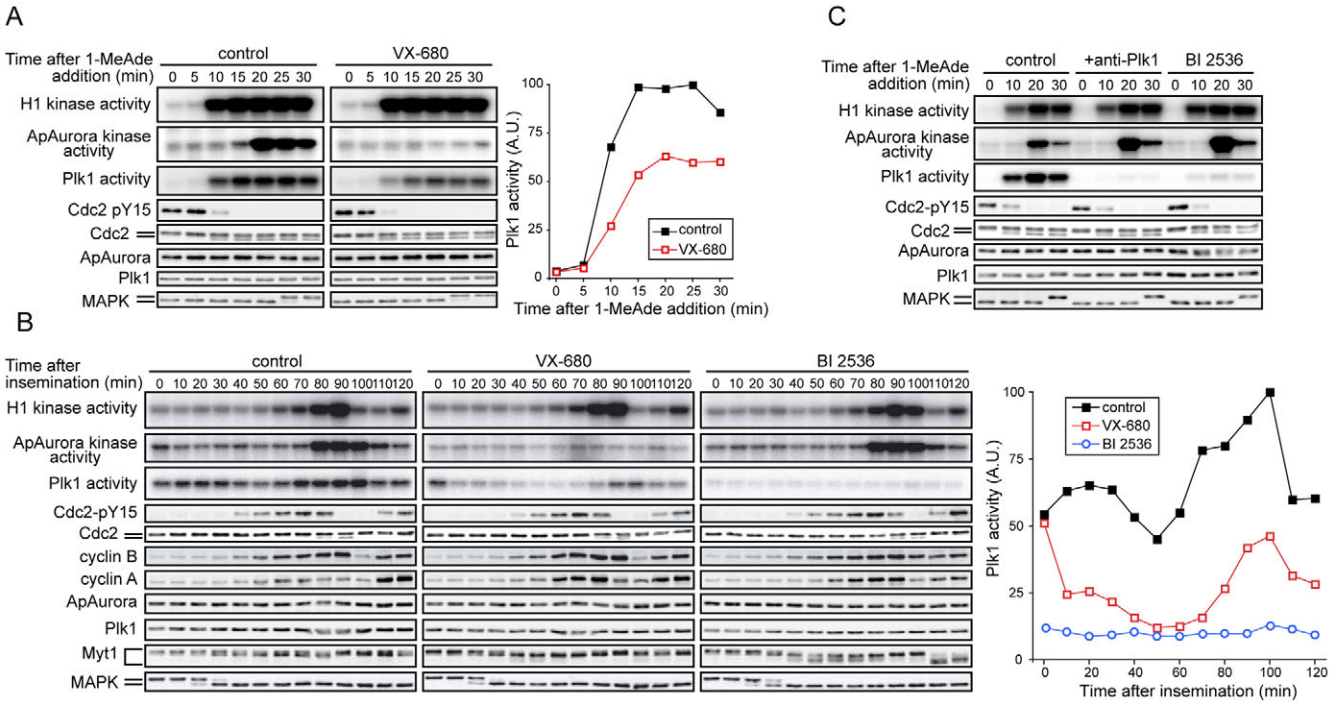

Fig. 3. Plk1 activation partially depends on ApAurora activity in starfish eggs. (**A**) ApAurora activity is not essential for, but contributes partially to Plk1 activation at reinitiation of meiosis I. Immature oocytes were preincubated with DMSO (control) or 2 µM VX-680. Quantified Plk1 activities are shown on the right-hand side. Shift-down of Myt1 bands in immunoblot supports inhibition of Plk1 activity in vivo (Okano-Uchida et al., 2003). (**B**) ApAurora activity is partially required for Plk1 activation, but not vice versa, in early embryos. Unfertilized mature eggs were treated with DMSO (left), 2 µM VX-680 (middle) or 2 µM BI2536 (right), followed by insemination. Quantified Plk1 activities are shown on the right-hand side. (**C**) Plk1 activity is not required for ApAurora activation at reinitiation of meiosis I. Immature oocytes were preincubated with 2 µM BI2536 or DMSO (control), or injected with neutralizing anti-Plk1 antibody (Okano-Uchida et al., 2003) (middle). Cell cycle progression was monitored by immunoblots using anti-Cdc2-pY15, anti-Cdc2, anti-cyclin A, anti-cyclin B, anti-Myt1 and anti-MAPK antibodies, and by H1 kinase activity.

ApAurora shows the phenotype of both Aurora-A and Aurora-B in starfish eggs

Because Aurora-A and Aurora-B have particular functions corresponding to distinctly different subcellular localizations, we first used immunofluorescence staining to examine whether ApAurora is localized as either Aurora-A or Aurora-B in starfish oocytes. Although the signal was slightly detectable in the GV of immature oocytes (Fig. 4A, 0 minutes), ApAurora protein levels did not show significant change in immunoblots even when GV was removed (see supplementary material Fig. S3), indicating that localization of ApAurora is essentially cytoplasmic in immature oocytes. After 1-MeAde addition, ApAurora accumulated in GV (Fig. 4A, 10 minutes) and, following GVBD, became localized on chromosomes (Fig. 4A, 20 and 30 minutes). Thereafter, ApAurora was localized both on chromosomes and meiotic spindle (Fig. 4A, 40 and 50 minutes), and then relocated from chromosome to midzone at anaphase onset (Fig. 4A, 60 minutes). In early embryonic M phase, ApAurora was localized both on chromosomes and mitotic spindle, and then relocated from chromosome to midzone (Fig. 4B). In contrast to meiosis I, however, considerable localization of ApAurora in spindle poles was observed in embryonic mitosis (Fig. 4B). ApAurora thus exhibits subcellular localization both as Aurora-A and Aurora-B in starfish meiotic and early embryonic cycles.

We next examined the defect in the formation of meiotic and mitotic apparatus caused by inhibition of ApAurora activity. Normally, dispersed chromosomes in GV congressed together following GVBD at meiotic reinitiation in control oocytes (Fig. 4C, 20 and 30 minutes) (Lenart et al., 2005); however, in VX-680-treated oocytes, uncongressed chromosomes were detectable and unusually separated aster-like spindles were formed (Fig. 4C). Despite this severe defect in meiotic apparatus formation, oocytes

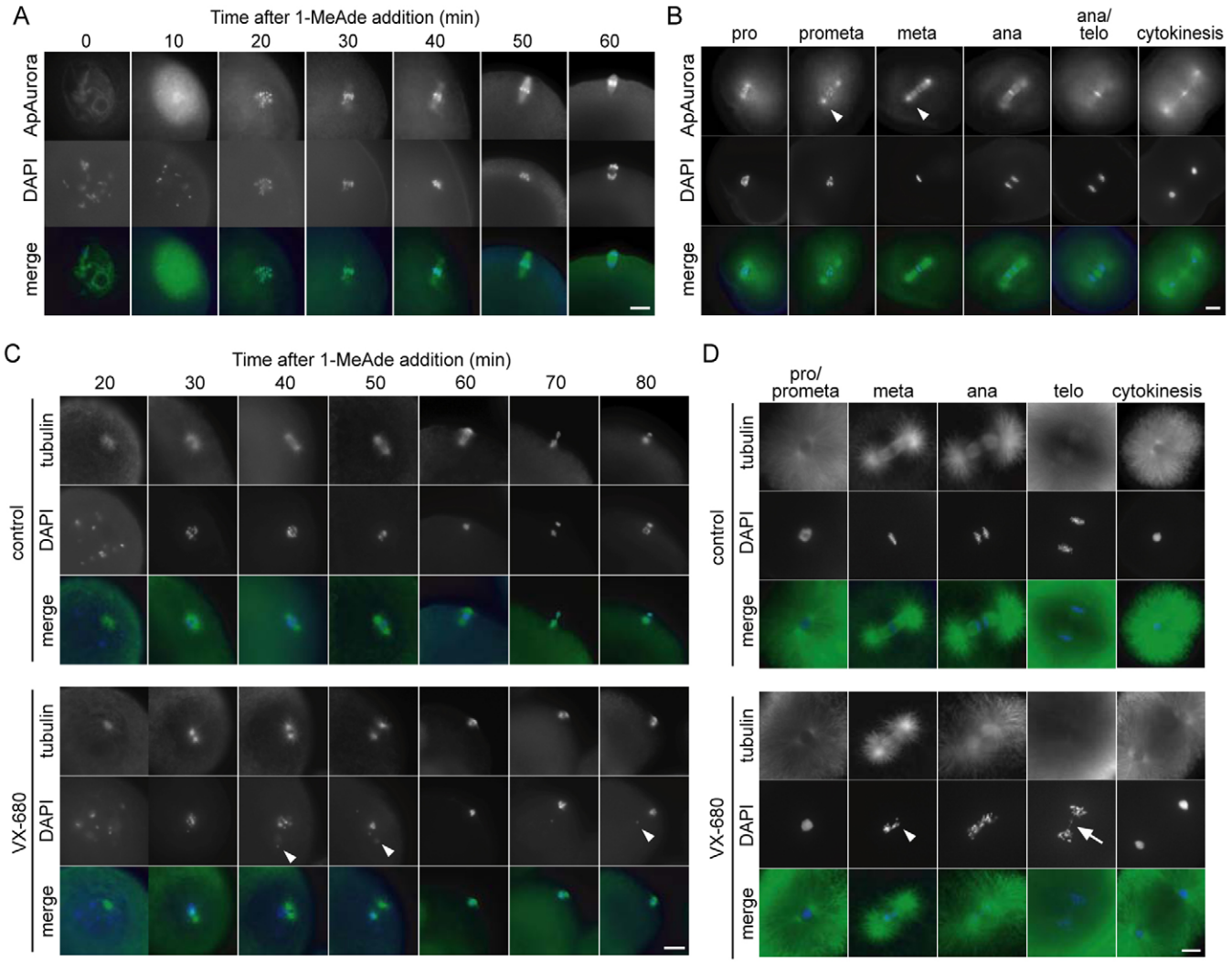

Fig. 4. ApAurora behaves as both Aurora-A and Aurora-B in starfish eggs. (**A**,**B**) ApAurora is localized on centrosome, spindle, chromosome and midbody as both Aurora-A and Aurora-B in meiosis I and early embryonic M phase. Oocytes prepared from meiosis I (A) or blastomeres from the first M phase (B) were stained with anti-ApAurora antibodies (green) and DAPI (blue). (**C**) ApAurora activity is required for accurate progression in meiosis I. Immature oocytes were preincubated with DMSO (control) or 2 μM VX-680, and then treated with 1-MeAde. Oocytes were stained with anti-tubulin antibodies (green) and DAPI (blue). Un-congressed chromosomes are indicated by the arrowhead. (**D**) ApAurora activity is required for accurate progression in early embryonic M phase. Maturing oocytes were inseminated at meta-I. After the second polar body extrusion, fertilized mature eggs were treated with DMSO (control) or 2 μM VX-680. Blastomeres were stained with anti-tubulin antibodies (green) and DAPI (blue). Misaligned chromosomes and lagging chromosomes are indicated by the arrowhead and arrow, respectively. Scale bars: 20 μm.

Table 1. HeLa cell lines used in this study

Transfection	Description
Mock	HeLa 'Kyoto', parent cell line
ApAurora^{WT}	Starfish *Asterina pectinifera* Aurora tagged with FLAG at its N-terminus
ApAurora^{KD}	ApAurora^{WT} with Asp290Ala mutation; kinase-inactive mutant

tried to force the first and second polar bodies into extrusion, but finally failed to form them (see supplementary material Fig. S4A and Movies 1–3). Next, to examine the effect of ApAurora inhibition on early embryos, maturing oocytes were inseminated at meta-I and then VX-680 was added at the second polar body extrusion. Although bipolar, but slightly smaller spindles, were detectable, the chromosomes were misaligned on the metaphase plate along with lagging chromosomes, and finally cleavage failure occurred (Fig. 4D; supplementary material Fig. S4B and Movies 4–6). Thus, in starfish meiotic and early embryonic cycles, inhibition of ApAurora activity causes the defects similar to those caused by downregulation both of Aurora-A and Aurora-B.

Taken together, the results indicate that ApAurora exhibits the subcellular localization and function of both Aurora-A and Aurora-B. Considering all the above observations, it is plausible that a single ApAurora could be the prototype both of Aurora-A and Aurora-B.

Localization of ApAurora in HeLa cells

To further test the possibility that ApAurora is the prototype of vertebrate Aurora-A and Aurora-B, we established HeLa cell lines in which wild-type ApAurora (ApAurora^{WT}) or its kinase-inactive mutant (ApAurora^{KD}) were stably expressed (Table 1 and Fig. 5A). Because HeLa cells expressing either ApAurora^{WT} or ApAurora^{KD} showed no mitotic defects (data not shown), we examined whether ApAurora localization was similar to that of Aurora-A and Aurora-B. In HeLa cells, ApAurora was localized on centrosomes, as is Aurora-A, but did not exhibit the localization patterns of Aurora-B on the inner centromere and midbody (Fig. 5B).

Because Aurora-B localization depends on formation of chromosomal passenger protein complex (Ruchaud et al., 2007), we examined the interaction between ApAurora and INCENP. Indeed, this interaction was almost prevented in the presence of Aurora-B (Fig. 5C). However, when Aurora-B was depleted by RNA interference (RNAi) in unsynchronized cells, ApAurora was found to strongly bind to INCENP (Fig. 5C). Consistently, in Aurora-B-depleted cells, ApAurora became detectable on the inner centromere and midbody (like Aurora-B) as well as in centrosomes (Fig. 5B, right). Thus, ApAurora can exhibit subcellular localization similar to that of both Aurora-A and Aurora-B in HeLa cells.

ApAurora restores the chromosome alignment defect and cytokinesis failure in Aurora-B depleted cells

In the above experiments (Fig. 5B), we noticed that HeLa cells stably expressing ApAurora^{WT} progressed through mitosis without detectable defects, even in the absence of Aurora-B. We then examined compensation of the Aurora-B function by ApAurora. When HeLa cells were depleted of Aurora-B by RNAi during a double thymidine block and then arrested at metaphase by treatment with the proteasome inhibitor MG132 (Fig. 6A,B), there was a severe defect in chromosome alignment on the metaphase plate. This chromosome misalignment by Aurora-B depletion was almost

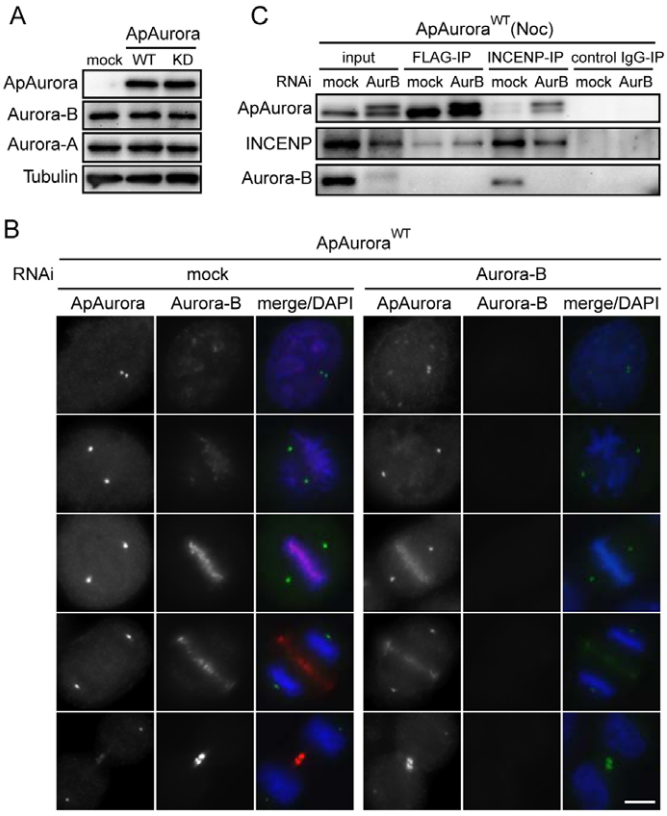

Fig. 5. Establishment of HeLa cell lines stably expressing ApAurora. (**A**) Expression of ApAurora^{WT} or ApAurora^{KD} in HeLa cells was confirmed by immunoblots. (**B**) Localization pattern of ApAurora in HeLa cells displays that of both Aurora-A and Aurora-B. HeLa cells stably expressing ApAurora^{WT} were transfected with Aurora-B siRNA (right) or mock siRNA (left), and then stained with anti-ApAurora (green) and anti-Aurora-B (red) antibodies, and DAPI (blue). Scale bar: 10 µm. (**C**) ApAurora interacts with HeLa cell INCENP. Mitotic cell lysates prepared from nocodazole-treated HeLa cells (Noc) were immunoprecipitated with anti-FLAG (ApAurora), anti-INCENP, or control rabbit IgG.

completely restored by expression of ApAurora^{WT}, but not ApAurora^{KD} (Fig. 6C,D). Moreover, Aurora-B depletion caused failure in cytokinesis and formation of multinucleated cells, whereas expression of ApAurora^{WT}, but not ApAurora^{KD}, reduced the rate of multinucleated cell formation (Fig. 6E). These observations indicate that the kinase-active ApAurora can substitute for the function of Aurora-B in HeLa cells.

ApAurora restores the bipolar spindle formation defect in Aurora-A-depleted cells

To examine whether ApAurora can restore the defect in bipolar spindle formation caused by Aurora-A depletion, we performed Aurora-A RNAi during double thymidine block in HeLa cells stably expressing ApAurora, and then collected mitotic cells after MG132 treatment (Fig. 7A,B). Under our experimental conditions, Aurora-A depletion frequently caused spindle defects (Fig. 7C,D). Expression of ApAurora^{WT}, but not of ApAurora^{KD}, significantly increased the rate of bipolar spindle formation, although the size of the restored bipolar spindle was slightly smaller than that in mock-transfected cells (Fig. 7C). Consistently, ApAurora was detectable in centrosome, but its separation was abortive in cells

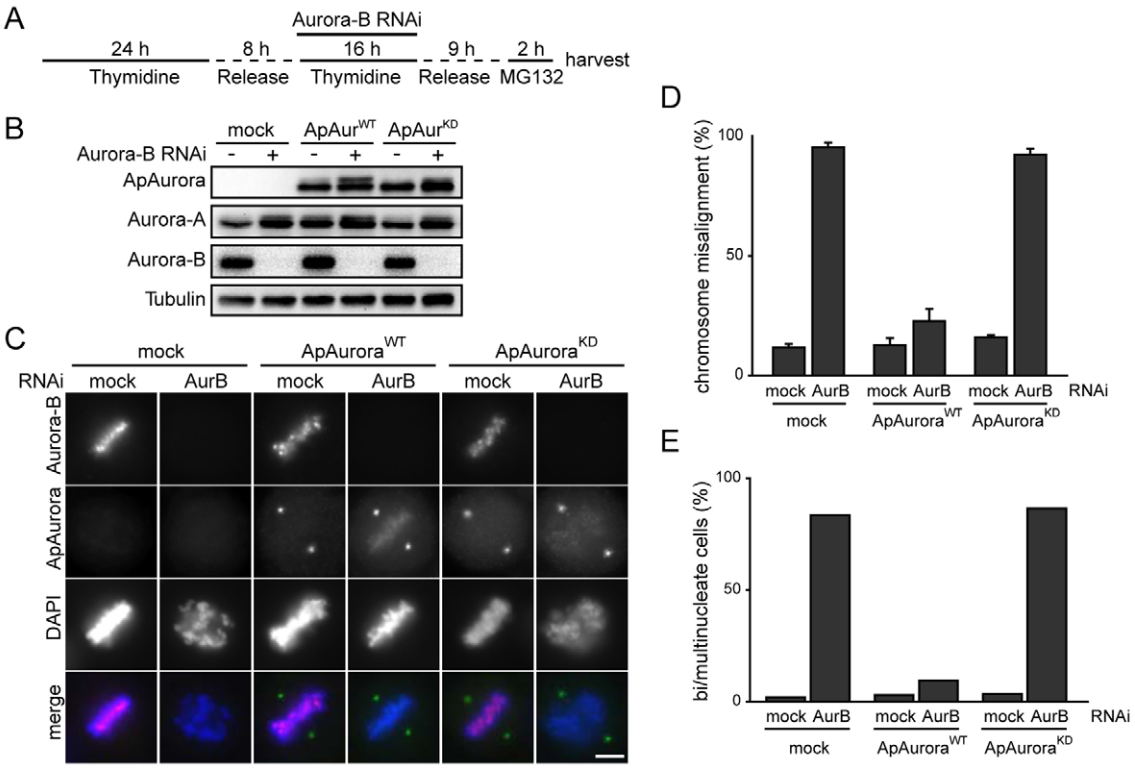

Fig. 6. ApAurora restores the chromosome alignment defects in Aurora-B-depleted HeLa cells. (**A**) Schematic overview of synchronization and RNAi treatment. HeLa cells of parent cell line or stably expressing either ApAurora^{WT} or ApAurora^{KD} were transfected with mock or Aurora-B siRNA during synchronization by the double-thymidine block, and then MG132 was added at G2–M transition. Two hours later, cells were analyzed by immunoblots or immunofluorescence. (**B**) Reduction of endogenous Aurora-B in HeLa cells was assessed by immunoblots. (**C**) ApAurora restores the defects by Aurora-B depletion in HeLa cells. Cells were stained with anti-ApAurora (green) and anti-Aurora-B (red) antibodies, and DAPI (blue). Scale bar: 10 μm. (**D**) Quantification of chromosome misalignment in C. Each data point represents three independent experiments by assessing 200 cells. Error bars represent s.d. (**E**) Quantification of rate of restoration by ApAurora in Aurora-B-depleted cells. Numbers of bi- and multinucleated cells were counted and summarized in a histogram (*n*=200 cells).

expressing ApAurora^{KD} (Fig. 7C). These observations indicate that the kinase-active ApAurora can restore the spindle formation defect in Aurora-A-depleted HeLa cells.

In these experiments, we failed to detect ApAurora localization on the mitotic spindle, in contrast to the clear localization of Aurora-A on it (Fig. 7C). Because spindle localization of Aurora-A depends on binding to TPX2, we examined the interaction between ApAurora and TPX2. Coimmunoprecipitation from HeLa cell extracts showed that ApAurora mostly did not bind to TPX2 either in the absence or presence of Aurora-A (Fig. 7E). Taken together, these observations suggest that ApAurora functions as Aurora-A for bipolar spindle formation, but in a different manner to Aurora-A, which depends on TPX2.

Discussion

In the present study, we isolated a cDNA for a starfish homolog of Aurora, and analyzed the properties of starfish Aurora protein (ApAurora) in starfish eggs and HeLa cells. Our cell biological observations show that in starfish oocytes and early embryos, the single ApAurora exhibits almost all the features of both Aurora-A and Aurora-B as regards subcellular localization and function (Figs 1–4); and that in HeLa cells, the single ApAurora of starfish can mostly substitute for both Aurora-A and Aurora-B of human (Figs 5–7). Similar ability of ApAurora was observed in double knockdown of HeLa cell Auroras (data not shown).

Localization and function of ApAurora in HeLa cells

When replacing Aurora-B in HeLa cells, ApAurora was able to bind to INCENP and almost completely compensated for the loss of Aurora-B (Figs 5, 6). These observations support the notion that Aurora-B function and INCENP are evolutionary conserved from yeast to human (Ruchaud et al., 2007).

By contrast, ApAurora failed to bind to TPX2, but rescued bipolar spindle formation in Aurora-A-depleted HeLa cells (Fig. 7). In this restoration experiment, ApAurora associated with centrosomes but not the spindle. This is consistent with a previous report that the interaction between Aurora-A and TPX2 is not required for bipolar spindle formation (Bird and Hyman, 2008). Rather, our observations indicate that association of Aurora, definitely ApAurora and possibly Aurora-A, with centrosomes is not mediated by TPX2, and that TPX2-independent Aurora function in centrosomes contributes to formation of the bipolar spindle, even though spindle length becomes short in the absence of TPX2-dependent Aurora function on microtubules.

In starfish eggs, however, ApAurora was localized not only on centrosomes but also on the spindle (Fig. 4A,B). Although it has been proposed that TPX2 does not have orthologs in invertebrates, excluding the nematode *C. elegans* (Ozlu et al., 2005), we isolated a starfish homolog of TPX2 (Y.A., unpublished; accession no. AB560815). The starfish TPX2 has a putative Aurora-A binding domain at its N-terminus corresponding to vertebrate TPX2, and is

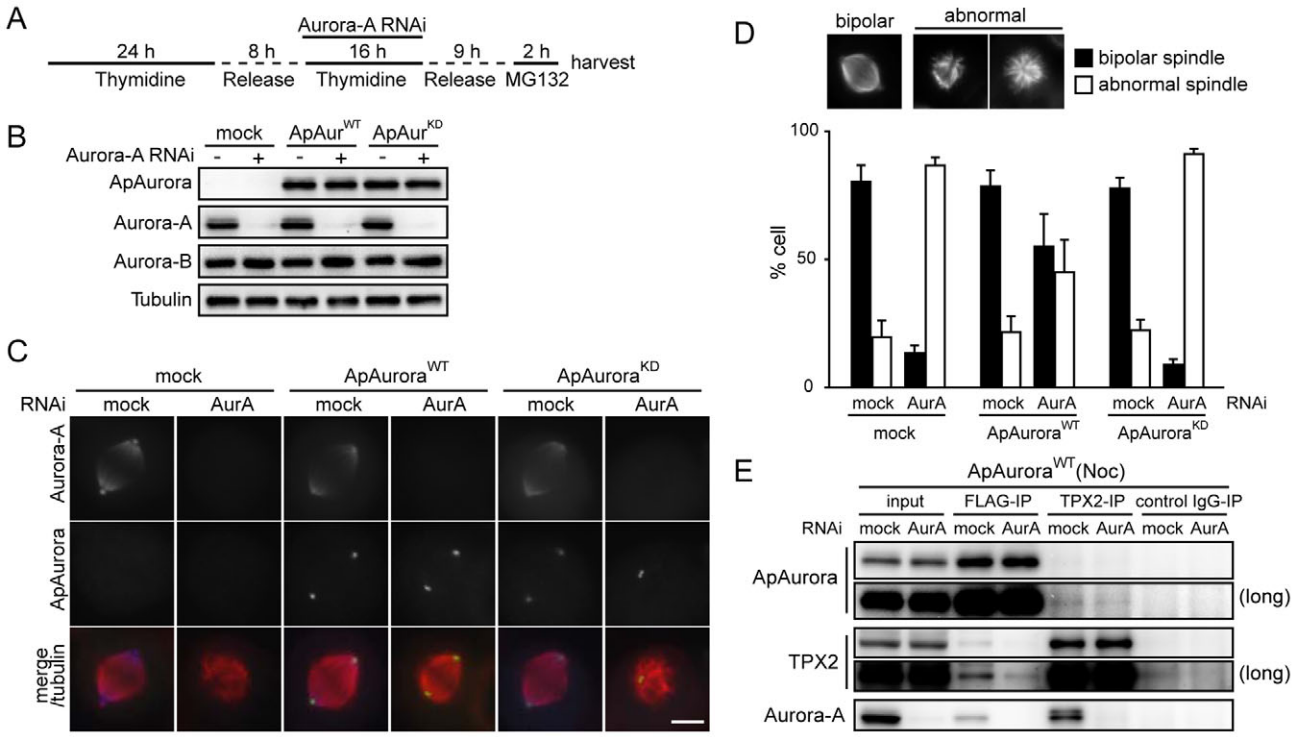

Fig. 7. ApAurora restores the bipolar spindle formation defect in Aurora-A-depleted HeLa cells. (**A**) Schematic overview of synchronization and RNAi treatment. (**B**) Reduction of endogenous Aurora-A in HeLa cells was assessed by immunoblots. (**C**) ApAurora restores the defects by Aurora-A depletion in HeLa cells. Cells were stained with anti-FLAG (ApAurora, green), anti-Aurora-A (blue), and anti-tubulin (red) antibodies. Scale bar: 10 µm. (**D**) Above: images of bipolar and abnormal spindles. Below: quantification of spindle morphology in C. Each data point represents three independent experiments, each assessing 200 cells. Error bars represent s.d. (**E**) ApAurora binds weakly to TPX2 in HeLa cells. Mitotic cell lysates prepared from nocodazole-treated HeLa cells (Noc) were immunoprecipitated with anti-FLAG (ApAurora), anti-TPX2, or control rabbit IgG. Coimmunoprecipitation of ApAurora with TPX2 was barely detectable even after long exposure (long).

detectable in immature oocytes (Y.A., unpublished). Hence, ApAurora in starfish eggs is likely to be localized on the spindle and to function in a TPX2-dependent manner, like Aurora-A in HeLa cells.

Interplay for activation of mitotic kinases

Activation of Plk1 requires phosphorylation on threonine within its activation T-loop (Liu and Maller, 2005), and we confirmed that this is also the case in starfish eggs (E.O., unpublished). Although the molecular identity of the T-loop kinase for Plk1 has been somewhat confusing (Archambault and Glover, 2009; Liu and Maller, 2005), two recent papers (Macurek et al., 2008; Seki et al., 2008) showed that it is Aurora-A that is mediated by association with the Bora–Plk1 complex. Consistent with this, we confirmed the existence of Bora protein in starfish eggs (Y.A., unpublished); Fig. 3A,B is evidence that full activation of Plk1 requires ApAurora activity. However, the same figures also demonstrate that this requirement is partial, i.e. Plk1 can be significantly activated in the absence of ApAurora activity, indicating that ApAurora is not essential for Plk1 activation. Rather, we previously demonstrated that Plk1 activation fully depends on cyclin-B–Cdc2 at reinitiation of meiosis I, and on cyclin-A–Cdc2 (and possibly also on cyclin-B–Cdc2 after entry into M phase) in early embryonic cycles (Okano-Uchida et al., 2003). With these considerations, a putative kinase downstream of Cdc2, but not ApAurora, is most likely to function as the T-loop kinase for Plk1. However, it remains elusive why this putative T-loop kinase alone can induce only partial, but not full, activation of Plk1.

Fig. 2 indicates that ApAurora activation fully depends on cyclin-B–Cdc2 in starfish eggs. Although the molecular mechanism of this dependence remains unclear, it might involve accumulation of ApAurora into nucleus (GV) at the activation (Fig. 4A). Conversely, even in the absence of ApAurora activity, cycling of cyclin-B–Cdc2 activity occurred almost normally throughout meiotic and early embryonic cycles. This independency could have become detectable due to lack of checkpoint response in the starfish egg system (Hara et al., 2009). Even so, this independency is apparently inconsistent with some previous reports indicating that Aurora-A positively regulates mitotic entry (Hirota et al., 2003; Liu and Ruderman, 2006; Macurek et al., 2008; Portier et al., 2007; Seki et al., 2008), and particularly with our previous study indicating that cycling of Cdc2 activity depends on Plk1 (Okano-Uchida et al., 2003) and Plk1 activity depends on Aurora (present study). However, considering that the dependency of Plk1 on Aurora is only partial (Fig. 3A,B), the inconsistency might be compromised, at least in starfish eggs, by assuming that a submaximum level of Plk1 activity, which is produced in the absence of Aurora, would be sufficient for activation of Cdc2.

Ancestral Aurora for vertebrate Auroras

Phylogenetic analyses indicate that the single type of Auroras of starfish, sea urchin and amphioxus weakly forms a clade (Fig. 8A).

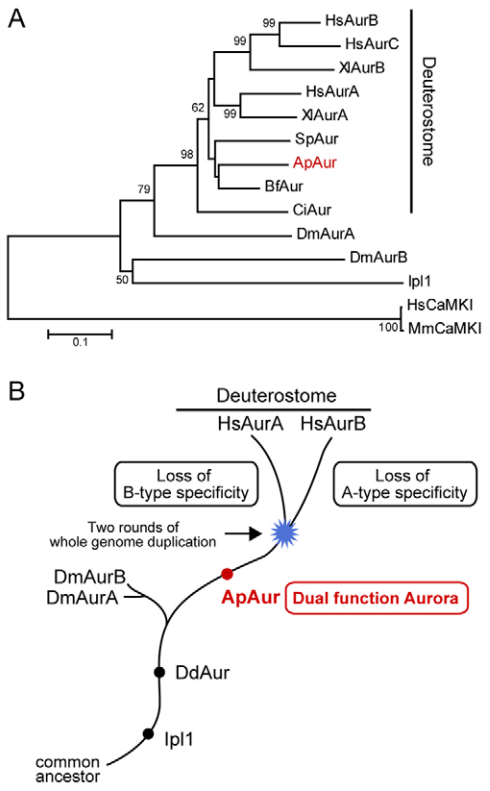

Fig. 8. Phylogenetic relationship and a model for Aurora kinase evolution. (**A**) Phylogenetic tree of Aurora kinases was generated by the neighbor-joining method based on the alignment of the catalytic domain. Bootstrap values over 50% are shown. Although ascidian Aurora (CiAur) does not group with the other non-vertebrate deuterostome Auroras in the phylogenetic tree, a similar tree was constructed by the maximum likelihood method or by the minimum evolution method. The anomaly in ascidian might be caused by its fast evolutionary rate (Putnam et al., 2008). Although the two fruit fly Auroras (DmAurA and DmAurB) do not form a monophyletic group, similar topology has been reported previously (Brown et al., 2004; Demidov et al., 2005; Li et al., 2008). Ap, *Asterina pectinifera*; Bf, *Branchiostoma floridae*; Ci, *Ciona intestinalis*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Sp, *Strongylocentrotus purpuratus*; Xl, *Xenopus laevis*. Ipl1 is the Aurora homolog of yeast *Saccharomyces cerevisiae*. The catalytic domain of CaM kinase I (CaMKI) was used for the out-group. The scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position. (**B**) In our model, vertebrate (Hs, human) Aurora-A and Aurora-B evolved, possibly via two rounds of whole genome duplication, from an ancestral single Aurora in non-vertebrate deuterostomes that gave rise to the single starfish ApAurora. Vertebrate Aurora-A and Aurora-B could have been specified by loss of the properties of the other type in the single dual-function Aurora. The single ApAurora originated from the common ancestor, via the single yeast Ipl1 and social amoeba DdAurora. Fruit fly Aurora-A and Aurora-B evolved after divergence of protostomes, independently of the evolution in vertebrates.

This clade is distinct from the vertebrate clade including Aurora-A, Aurora-B and Aurora-C, but forms a higher order clade of deuterostomes together with vertebrates and ascidian. Such a structure of the phylogenetic tree supports the idea that ApAurora in starfish represents the prototype for both Aurora-A and Aurora-B in vertebrates (Fig. 8B). Taking cell biological and phylogenetic analyses together, it is most likely that the single type of Aurora found in non-vertebrate deuterostomes (such as starfish, sea urchin, ascidian and amphioxus) represents the ancestor that gave rise to vertebrate Auroras. The ancestral single Aurora gene might have evolved into Aurora-A and Aurora-B during vertebrate evolution, possibly due to two rounds of whole genome duplication that occurred after the divergence of cephalochordates (Putnam et al., 2008).

How then did Aurora-A and Aurora-B in nematode or fruit fly evolve? Considering that they are paralogous to vertebrate counterparts (Brown et al., 2004), they might have evolved after the divergence of protostomes, independently of Aurora evolution in vertebrates (Fig. 8B). If so, a possible question would be why an independent separation of the single Aurora to both types resulted in functionally equivalent separation. One explanation would be to simplify the complexity of Aurora functions in the centrosome–spindle and inner centromere–midbody by division of labor between two variants of Aurora.

Recently, Fu et al. (Fu et al., 2009) and Hans et al. (Hans et al., 2009) showed that a single amino acid change within the Aurora-A kinase domain (Gly198 to the equivalent Asn residue of Aurora-B) is sufficient to convert it into an Aurora-B-like kinase in HeLa cells. The equivalent residue in ApAurora (supplementary material Fig. S1), and also in the single Auroras of sea urchin, ascidian, and amphioxus, is Gly instead of Asn, indicating that the single Aurora ancestral to vertebrates is classified to the A-type in the context of Gly198, but nevertheless can also behave as the B-type. In other words, the A-type, rather than the B-type, might be closer to the single ancestor. Consistent with this consideration, Aurora-A can bind with INCENP, at least in vitro (Fu et al., 2009). Thus, some property of the B-type is probably conserved from the single ancestral Aurora to vertebrate Aurora-A. This implies that specification to the A-type could have been caused by loss of the B-type property in the single ancestor, possibly through specific anchoring of Aurora-A by TPX2, which prevents its binding with INCENP as previously suggested (Fu et al., 2009).

How has the B-type been specified from the single ancestor? In the context of the Gly198 residue in the single ancestral Aurora and in the A- and B-type Auroras, a single amino acid change from Gly to Asn could be a major cause of the B-type specification (Fu et al., 2009; Hans et al., 2009). However, considering that the single ancestral ApAurora can exhibit the combined functions of the A- and B-types even though the Gly198 residue remains unchanged, the change from Gly to Asn during evolution of vertebrate Aurora-B might have contributed to loss of specificity of the A-type rather than to a gain of specificity of the B-type. Furthermore, the Aurora-B Asn142Gly (equivalent to Aurora-A residue) mutant is not able to bind to TPX2 in HeLa cells (Hans et al., 2009), implying that specification to the B-type does not simply depend on the Gly to Asn change. There might be an additional element(s) that specifies the B-type.

In conclusion, specification to the A-type and the B-type Auroras during vertebrate evolution could have been caused mainly by loss of the properties of the other type in the single ancestral Aurora with dual functions.

Materials and Methods

Oocytes, embryos and extracts

Immature oocytes of the starfish *A. pectinifera* were treated with 1 μM 1-MeAde to induce maturation. Insemination was performed during meiosis I or after completion of meiosis II. Microinjection into oocytes and preparation of oocyte or egg extracts for immunoblot and kinase assay were performed as described previously (Okano-Uchida et al., 2003).

Starfish Aurora cDNA cloning

To isolate starfish *A. pectinifera* Aurora cDNA fragments, degenerate PCR primers were designed from the highly conserved peptides FEIGRPL and DLISRLL. First

strand cDNA was synthesized from total RNA of immature oocytes using Superscript II (Gibco). PCR was performed with degenerate primers, and high sequence homology with Aurora was confirmed in PCR products. Full-length Aurora cDNA was isolated with the SMART RACE cDNA amplification kit (Clontech). The DDBJ accession number for Aurora sequence of the starfish *A. pectinifera* (renamed *Patiria pectinifera* in the 2007 NCBI Taxonomy Browser) is AB530259.

Antibodies and kinase assay

Anti-*Asterina* Aurora (ApAurora) rabbit polyclonal antibodies were raised against recombinant His-tagged Aurora (1–126 amino acids, unconserved region). Anti-ApAurora antibodies were affinity-purified with the antigen. For the ApAurora kinase assay, 10 μl of egg extracts equivalent to ten oocytes or fertilized embryos were prepared to perform immunoprecipitation (Okano-Uchida et al., 2003) with anti-*Asterina* Aurora antibody. Immunoprecipitated ApAurora was mixed with myelin basic protein (0.5 mg/ml) as a substrate in the presence of 20 μM roscovitine (Sigma-Aldrich) and 0.2 mCi/ml [γ-^{32}P]ATP, and the mixture incubated for 40 minutes at 26°C. Other antibodies used were: anti-*Asterina* cyclin A, anti-cyclin B, anti-Cdc25, anti-Myt1, and anti-Plk1 (Okano-Uchida et al., 2003); anti-MAP kinase (Upstate); anti-PSTAIR (Cdc2); anti-phospho-histone H3-Ser10 and anti-phospho-Cdc2-Tyr15 (Cell Signaling Technology); anti-Mcm2 (BD Biosciences); and anti-tubulin mouse mAb (B-5-1-2, Sigma Aldrich, used in immunoblots for HeLa cells). Plk1 and histone H1 kinase assays were performed as described (Okano-Uchida et al., 2003). Kinase activity was quantified by densitometric measurement of autoradiograms with Image Gauge software (Fuji Film). Typical data are shown as a representative of at least three independent experiments.

Kinase inhibitors

Aurora kinase inhibitor VX-680 was synthesized according to the published procedure (Tyler et al., 2007). BI 2536 (Axon Ligands), Roscovitine (Sigma-Aldrich) were purchased.

Phylogenetic analysis

Aurora homologs of *S. purpuratus* (XP_001181990), *C. intestinalis* (XP_002119314) and *B. floridae* (XP_002587531) were obtained by BLASTP search. The sequences were aligned using the CLUSTALW in BioEdit, and the alignment was adjusted by hand. After removing gaps, the verified alignment was used to construct phylogenetic trees. The tree was calculated using the MEGA program based on the neighbor-joining method.

Live cell imaging

Recordings were made at 20°C. Time-lapse images were collected at 30 seconds (for cleavage) or 60 seconds (for polar body extrusion) intervals with an Axiophot microscope (Zeiss) equipped with a Penguin 600CL camera that was driven by StreamPIX software (Pixera). The images were processed using ImageJ software (National Institute of Health).

Cell culture and transfection

HeLa cells were cultured in DME supplemented with 10% FCS, 0.2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 environment. To generate HeLa cell lines that stably express ApAurora, HeLa cells were transfected with pIRESpuro3-ApAurora of full-length wild-type or kinase-inactive mutant (Asp290Ala), and cultured in the presence of 0.25 μg/ml puromycin for more than 2 weeks. Stable expressants were screened by immunoblot. For RNAi of Aurora-A or Aurora-B, the targeted sequences were as follow: Aurora-A, 5′-UCAGCGGGUCUUGUGUCCUUCAAAU-3′; Aurora-B, 5′-AACAUCCUGCG-UCUCUACAACUAUU-3′ (Stealth; Invitrogen). Transfections were carried out by incubating HeLa cells with 50 nM siRNA oligonucleotides and RNAi MAX (Invitrogen) in the absence of antibiotics during the cell synchronization regimen. For control, the same procedure was set up using H_2O.

Immunoprecipitation from HeLa cell lysates

Mitotic HeLa cells were prepared by arrest with 100 ng/ml nocodazole after release from double thymidine block. Cells were lysed in a lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 20 mM β-glycerophosphate, 5 mM $MgCl_2$, 5% glycerol, 0.1% NP40). The lysate was incubated with protein A–Sepharose conjugated with anti-FLAG (M2, Sigma-Aldrich), anti-TPX2 (Novus Biologicals), or anti-INCENP (gift from Jan-Michael Peters, Research Institute of Molecular Pathology, Vienna, Austria) for 1 hour at 4°C. The beads were washed three times with lysis buffer and run on SDS-PAGE followed by immunoblot.

Immunofluorescence

Starfish eggs

Immunofluorescence was performed as described (Tachibana et al., 2008). Oocytes or embryos were fixed by cold methanol for 15 minutes, blocked by PBS with 5% skimmed milk for 1 hour, and then incubated overnight at 4°C with anti-tubulin rat mAb (MCA77G, Serotec) diluted at 1:200 or 0.2 μg/ml anti-ApAurora in PBS with 5% skimmed milk. Secondary antibodies were donkey anti-rat IgG Alexa-Fluor 488 and donkey anti-rabbit IgG Alexa-Fluor 488 (Molecular Probes) in PBS with 5% skimmed milk. After washing with Triton-X-containing PBS, the specimens were stained for 30 minutes with 0.1 μg/ml DAPI. Images were captured with an Axioplan2 microscope equipped with an AxioCam camera driven by AxioVision software (Zeiss).

HeLa cells

Cells were fixed by cold methanol for 10 minutes, and then blocked by 0.01% Triton X-100 in PBS with 3% BSA for 1 hour. Triple immunofluorescence staining with ApAurora, Aurora-B, and DNA was performed as follows: Cells were incubated with 0.2 μg/ml rabbit polyclonal anti-ApAurora antibodies, followed by incubation with mouse monoclonal anti-Aurora-B antibodies (BD Biosciences). After washing with PBS, the primary antibodies were probed with goat anti-rabbit IgG Alexa-Fluor 488 and goat anti-mouse IgG Alexa-Fluor 568 (Molecular Probes). DNA was then stained with 0.1 μg/ml DAPI.

Triple immunofluorescent staining with ApAurora, Aurora-A, and tubulin was performed as follows: Cells were incubated with mouse monoclonal anti-FLAG antibodies (M2, Sigma-Aldrich), followed by incubation with rabbit polyclonal anti-Aurora-A antibodies (Trans Genic), and then incubated with rat monoclonal anti-tubulin antibodies (YL1/2, Chemicon). After washing with PBS, the primary antibodies were probed with goat anti-mouse IgG Alexa-Fluor 488, goat anti-rabbit IgG Alexa-Fluor 350, and goat anti-rat IgG Alexa-Fluor 568 (Molecular Probes). After staining, cells were mounted with Fluorescent Mounting Medium (Dako Cytomation). Images were captured with an AxioImagerM1 microscope (Zeiss) equipped with a CoolSNAP HQ camera (Photometrics) driven by MetaMorph software (MDS Analytical Technologies).

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http://jcs.biologists.org/cgi/content/full/123/22/3978/DC1

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